

Rapid Biosynthesis Method and Characterization of Silver Nanoparticles Using *Zizyphus spina christi* Leaf Extract and Their Antibacterial Efficacy in Therapeutic Application

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Abstract

Antibacterial activity of biosynthesized silver nanoparticles (AgNPs) was significant in therapeutic application of nanotechnology. These researchers studied an eco-friendly and rapid method for the first time to synthesize silver nanoparticles using *Zizyphus spina christi* L aqueous leaves extract (ZSE), and their antibacterial properties. The extract was found to have the potential to form silver nanoparticles at room temperature within few minutes. The green synthesized silver nanoparticles were characterized using different techniques. The UV-visible spectrum of the solution containing AgNPs showed a peak at 414 nm corresponding to the plasmon absorbance of silver nanoparticles. The transmission electron microscopy (TEM) showed that the formed particles were hexagonal in shape with appreciable Nano size ranging from 21.5 to 59.67 nm. Fourier Transform Infrared Spectroscopy analysis (FTIR) of biosynthesized AgNPs affirmed the role of ZSE as reducing and capping agent of Ag⁺ ions to AgNPs, and X-Ray Diffraction patterns (XRD) showed that they could be indexed as face-centered-cubic structure of silver. Antibacterial activity of AgNPs was determined by well diffusion and micro plate assay methods, showing maximum inhibition zones of 24 mm, 23 mm, 15 mm and 17 mm against *Staphylococcus aureus*, *Acinetobacter sp.*, *Pseudomonas aeruginosa* and *Escherichia coli* respectively. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) results showed that AgNPs had MIC, MBC of 45, 57 µg/mL, 49, 61 µg/mL, 63, 90 µg/mL and 59, 82 µg/mL against *S. aureus*, *Acinetobacter sp.*, *P. aeruginosa* and *E. coli* respectively. Furthermore, the green synthesized AgNPs were loaded on band-aids and screened for antibacterial activity. The AgNPs loaded on band-aids exhibited strong antibacterial effect against multi drug resistant bacteria. These nanoparticles could be used for treating wounds and preparing wound dressing. Such researches are crucial in the demonstration of therapeutic importance of silver nanoparticles in medical application.

Keywords

Silver Nanoparticles, *Zizyphus spina christi* L, Antibacterial Activity, Therabiotic

1. Introduction

Synthesis of nanoparticles has gained great significance during the last few years due to their unique properties and application [1]. Chemical methods are among the most important approaches in metallic nanoparticles synthesis. However, these methods use high cost and toxic reagents as reducing and stabilizing agents [2]. Currently, there is a growing need to develop inexpensive and environmentally friendly nanoparticles synthesis processes that do not use toxic chemicals in the synthesis protocol [3]. Today, synthesis of nanoparticles is carried out mainly on biological systems such as bacteria [4], fungi [5], yeast [6], algae [7] and plant extracts [8]. In microorganism-mediated methods, the synthesis reaction takes a long time (24 - 124 h) and the process of sub-culturing cell cultures is time-consuming [9] [10], whereas in plant-mediated methods, reaction time is greatly short and cultivation of microbial cells step is eliminated, also no need to focus on cross contamination as like microbes.

Although biosynthesis of nanoparticles using plant extracts has already been reported in various plants such as neem [11], *Aloe vera* [12], *Cinnamomum camphora* [13], *Rosa hybride* [14] and *Mulberry leaf* [15], there is still a lot of attention to this field because of the diversity and the high potential of plants as reducing agent in producing nanoparticles with different shapes. There are many solvents used for extracting the plant components for nanoparticles synthesis like methanol [15] ethanol [16], diethyl ether, chloroform, ethyl acetate [17] and water [18] [19] [20]. As the research is focused on eco-friendly and low cost methods for synthesis of nanoparticles, it is always preferable to use water (aqueous solvent) for extraction procedures and preventing the use of toxic chemicals. Moreover, water is high polar solvent and it is very simple to extract the phytochemical compounds from plant leaves into medium. Recently, researchers have reported that the water soluble organic compounds present in the plant leaves extracts are found to be strong reducing agents for nanoparticles synthesis [21]. The water soluble organics and phenolics present in the plant extracts are mainly reducing agents to change silver ions to metallic AgNPs [22]. Phytochemical analyses of *Zizyphus spina christi* L leaf extract revealed the presence of phenolic, tannins, flavonoids, alkaloids and saponins. These phytochemical compounds in *Zizyphus spina christi* L. are strong reducing agents due to their abundant OH-groups that enhance their antibacterial and antioxidant activity [22].

Hence, this research work is mainly focused on simple process as a green technology using aqueous extract of *Zizyphus spina christi* L leafs as a first time for the biosynthesis of silver nanoparticles without the usage of hazardous and toxic solvent. The process has several advantages with low cost, compatibility, stability and also has proved their medical application as wound dressing against skin infection pathogen.

2. Methodology

2.1. Collection, Processing and Preparation of *Zizyphus spina christi* Leaf Extract

Fresh leaves of *Zizyphus spina christi* L were collected from Taif, KSA. The leaves were thoroughly washed under tap water to remove the adhered and then rinsed with Milli-pore-Milli-Q water. The aqueous leaf extract of *Zizyphus spina christi* L was prepared as follows: 25 g of fresh leaves were mixed with 100 mL of distilled water in a 250 mL flask and the contents were boiled for 10 min. The components were cooled to room temperature and filtered through Whatman No. 1 filter paper. The clear leaf extract of *Zizyphus spina christi* L thus obtained was used for synthesis of silver nanoparticles.

2.2. Synthesis of Silver Nanoparticles

Ten milliliter of 25% *Zizyphus spina christi* L aqueous extract (ZSE) was gradually added into 90 ml of 1 mM silver nitrate (AgNO_3) in a 250 ml Erlenmeyer flask and incubated at room temperature with shaking under dark conditions. The reaction solution was checked for 30 minutes and monitoring the change in color of AgNO_3 solution from colorless to brown. The AgNPs solution was centrifuged at 15,000 g for at least 30 min. The supernatant was discarded and obtained pellet was redispersed in deionized water. The centrifugation for pellet was repeated two to three times to wash off any substances on the surface of silver nanoparticles.

2.3. Characterization of AgNPs

2.3.1. UV-Vis Spectra Analysis

Analysis of UV-Vis spectra was done by using a double beam spectrophotometer (Shimadzu uv-1650 pc spectrophotometer) [23]. The solution of silver nanoparticles was monitored by measuring the UV-Vis spectrum of the reaction solution in the 300 - 900 nm wavelength rang after diluting a small aliquot of the solution into deionized water. The solution was pipetted into a test tube and diluted four times by deionized water and analyzed at room temperature.

2.3.2. Transmission Electron Microscope (TEM)

The AgNPs was analyzed by using transmission electron microscope (JEOL 100CX II with an accelerating voltage of 100 kV, Japan). Silver nanoparticles for TEM analysis was prepared by coating of aqueous solution of AgNPs drops on carbon-coated copper grids, and left to dry for 5 min; the extra solution was removed using blotting paper at room temperature [24].

2.3.3. Fourier Transform Infrared Spectroscopy (FT-IR)

Analysis of FT-IR spectra were recorded in the $4000 - 400 \text{ cm}^{-1}$ with a Shimadzu IR-470 Spectrometer (Shimadzu, Japan), equipped with data station. Dried samples of about 100 mg were mixed with 100 mg of spectral grade KBr and pressed into discs under hydraulic pressure [25].

2.3.4. X-Ray Diffraction (XRD)

X-ray diffraction analysis was detected to examine the crystallographic structure of the

purified AgNPs [26]. The XRD grids were coated with dried biosynthesized nanoparticles and the synthesized nanoparticles diffraction pattern was measured by X-ray diffractometer (Shimadzu XD-3A).

2.4. Antibacterial Activity

2.4.1. Determination of Antibacterial Activity of AgNPs

The AgNPs and aqueous extract prepared by *Zizyphus spina christi* L were used to evaluate antimicrobial activity against Gram positive bacteria, *Staphylococcus aureus* (*S. aureus*) and Gram negative bacteria, *Escherichia coli* (*E. coli*), *Acinetobacter sp.* and *Pseudomonas aeruginosa* (*P. aeruginosa*) on Mueller Hinton Agar plates by agar well diffusion method [27]. The Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) methods for all test bacterial strains were also determined using micro plate assay method.

2.4.2. Antimicrobial Activity of AgNPs-Band Aids

One hundred microliter of AgNPs was added on the sterilized band aids of 1 cm² under sterilized condition. The band aids was kept at 50°C for overnight to evaporate the excess water content and tested against the four multidrug resistant pathogenic bacteria (*S. aureus*, *Acinetobacter sp.*, *P. aeruginosa* and *E. coli*). The pathogenic bacteria were spread on Mueller Hinton Agar plates and AgNPs-band aids were placed on the surface of agar. The petri dishes were then incubated at 37°C for 24 hours. Finally, the zone of inhibition was determined.

3. Results and Discussion

3.1. Biosynthesis of AgNPs

The formation of silver nanoparticles was monitored with color change and UV-Vis spectroscopy. The biosynthesis of AgNPs was prepared using 90 ml of AgNO₃ solution and 10 ml of *Zizyphus spina christi* L leaf extract 25%. Change in color of solution from colorless to deep brown was observed for the formation of silver nanoparticles in the solution after 30 min of incubation at room temperature and shaking condition (Figure 1), indicating the formation of AgNPs [28].

3.2. Characterization of Silver Nanoparticles

3.2.1. UV-Vis Spectra Analysis

Noble metals are known to display unique optical properties due to the property of surface plasmon resonance (SPR). The reduction of AgNO₃ to silver nanoparticles by the leaf extract of *Zizyphus spina christi* L was confirmed by measuring the UV-Vis spectrum of the solution. The silver nitrate solution (1 mM) was added to the aqueous leaves extract (25%). This change of color was due to the reduction of Ag⁺ to Ag⁰ via the active biomolecules present in the leaves extract such as alcohol, phenols polysaccharide and protein [28]. On the other hand higher extract concentrations of the biomolecules act as reducing agent and cap the nanoparticle surfaces and protect them from aggregation. Similar studies showed that a different component in the extract is responsible for the synthesis of symmetrical nanoparticles [28] [29]. The brown color is caused to the excitation of surface plasmon resonance (SPR). As shown in Figure 2, a

characteristic and well-defined SPR band for silver nanoparticles was confirmed at around $\lambda = 414$ nm. The absorption band was constant for more than 3 months which substantiated the stability of the nanoparticles (data not shown).

3.2.2. Transmission Electron Microscope (TEM)

The size and morphology of AgNPs were studied by the transmission electron microscopy (TEM) images, by using the JEOL TEM 100 CXII with an accelerating voltage of 120 kV (Electron Microscope Unit, Assiut University, Egypt). The TEM grids were prepared by placing a drop of the reduced solution on a carbon-coated copper grid and by later drying it in air. Smaller sized mixture of triangle and cubic shape nanoparticles were obtained at the operational temperature. There is a variation in particle sizes from 21.5 to 59.67 nm (**Figure 3**). These particles were separated and are not aggregated. These small-sized nanoparticles were able to penetrate across the membrane [30] [31] [32]. It was evident that the silver nanoparticles edges were brighter than the center of the nanoparticles, suggesting the particles were encapsulated by biomolecules such as proteins in the *Ziziphus* leaf extract as confirmed by other studies [29] [30] [31] [32] [33].

3.2.3. XRD Analysis

The crystalline of silver nanoparticles was confirmed by the analysis of XRD pattern.

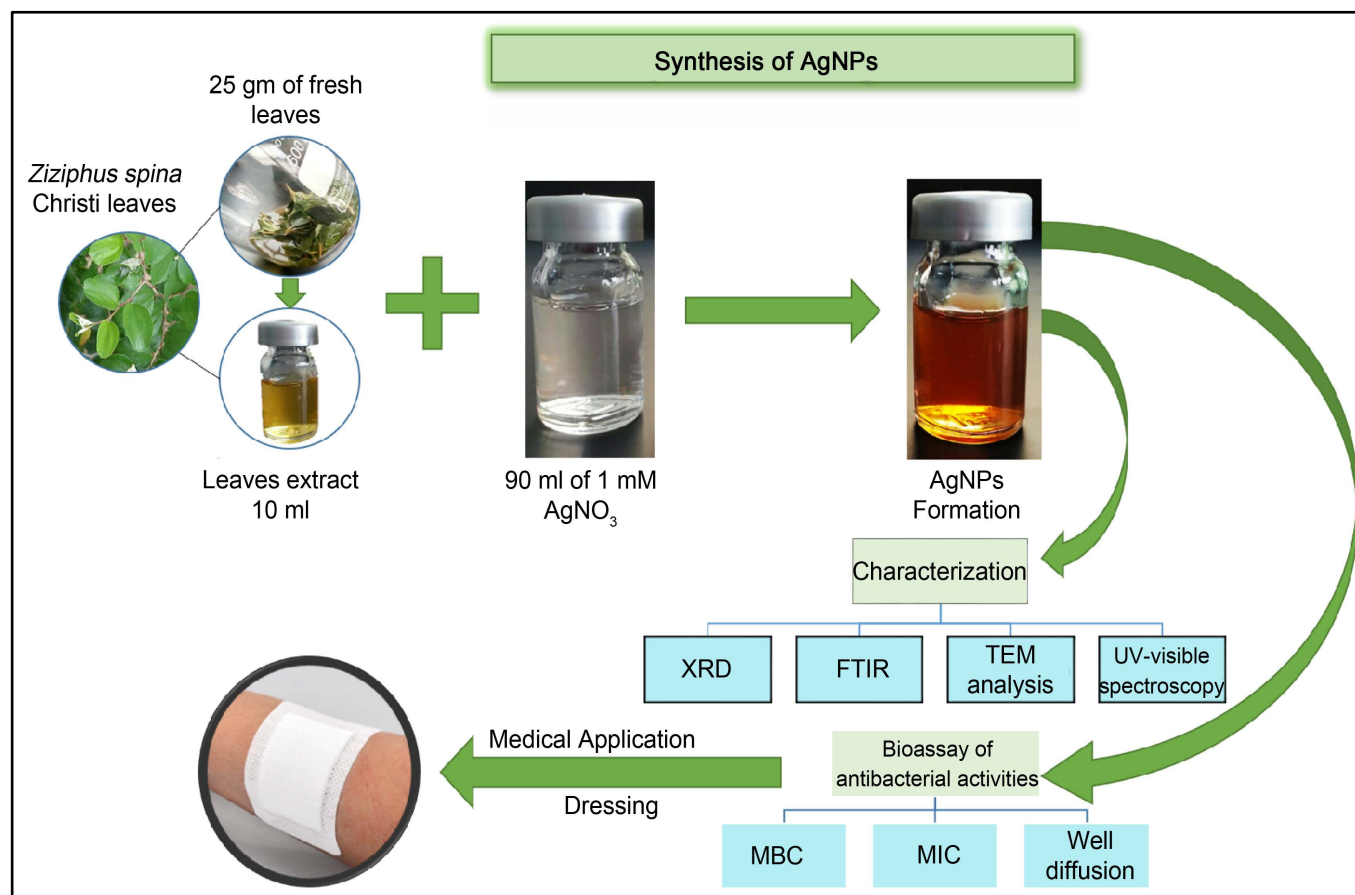


Figure 1. Schematic presentation for biosynthesis, characterization and biomedical applications of synthesized silver nanoparticles using *Ziziphus spina-christi* L aqueous extract.

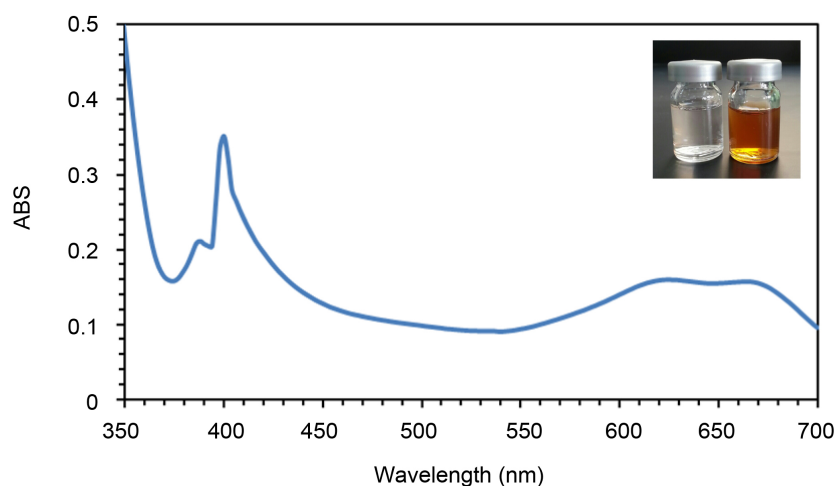


Figure 2. UV-vis spectra of AgNPs (90 ml 10^{-3} M AgNO_3 and 10 ml *Zizyphus spina christi* L leaf extract 25%).

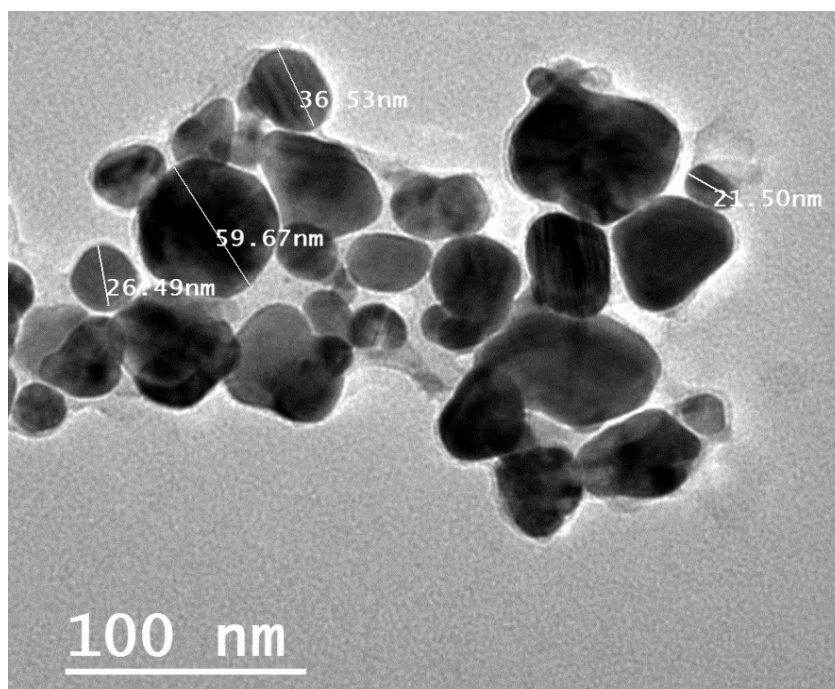


Figure 3. TEM micrograph of the silver nanoparticles after bioreduction (Ag^+ to Ag^0) by *Zizyphus spina christi*.

Figure 4 shows typical XRD pattern of the AgNPs prepared with 25% *Zizyphus spina christi* L extract and the AgNO_3 concentration 1 mM. Many distinct diffraction peaks at approximately 38.09° , 46.11° , 64.51° and 74.77° were assigned to reflections from the 2 theta region, corresponding to (111), (200), (220), and (311) planes of silver respectively. This confirms the basis that they can be displayed as triangle and face-centered-cubic (FCC) structures of silver; it suggested that the synthesized silver nanoparticles were biphas in nature [29]. Additional peaks were also observed at 32.15° , 54.73° , and 57.41° . They are apparent in many works in which the XRD pattern included the relevant 2θ range. These peaks were due to the organic compounds that were present the extract

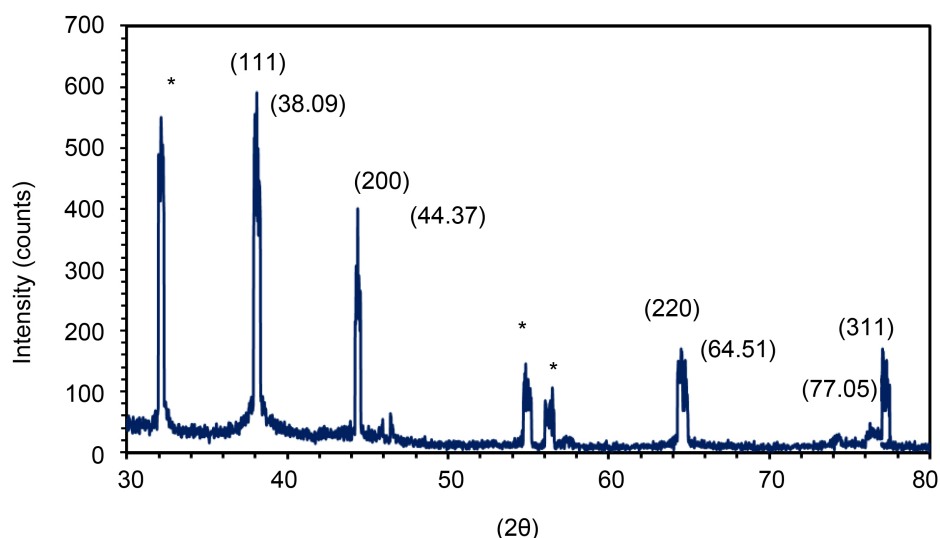


Figure 4. X-ray diffraction pattern of Ag nanoparticles prepared with aqueous *Zizyphus spina christi* L extract, * Peaks reveal to organic compound of extract.

and responsible for silver ions reduction and stabilization of resultant nanoparticles, similar results were reported earlier [33].

3.2.4. Fourier Transform Infrared Spectroscopy (FTIR)

FT-IR analysis measurements were carried out to identify the possible biomolecules responsible for the reduction of the Ag^+ ions and the capping of AgNPs synthesized by the extract of *Zizyphus spina christi* L. The solution after complete reduction was centrifuged at 15,000 rpm for 10 minutes to isolate the AgNPs free from the compounds present in the solution. FTIR spectrum of biosynthesized AgNPs showed absorption peaks at 3451.58, 2929.981, 1600.95 and 1380.80 cm^{-1} (Figure 5). Strong absorption peak at 3451.58 cm^{-1} was resulted from stretching of the O-H groups due to the presence of alcohols, phenols, carbohydrates, and etc., while the peak of 1380.80 cm^{-1} indicating to carboxyl groups. Some works observed modification of these bands after silver recovery with inactivated plant tissue, plant extract and living plant, depend on the natural biomolecules in plants [32] [34]. The variations of the hydroxyl and carboxylate groups have been reported in the several studies on synthesis of AgNPs with another polysaccharide [35] [36]. The peak that appeared around 2921 cm^{-1} is related to the stretching of the C-H bonds [37]. The peak at around 1600 cm^{-1} was assigned to the amide I bonds (NH) of proteins. Additional absorption peaks at 1727.222 and 1075.71 cm^{-1} were influenced by the presence of the AgNPs.

3.3. Antimicrobial Assay

The antibacterial effect of biosynthesized AgNPs was studied against multi drug resistant (MDR) bacteria in gram positive (*S. aureus*) and gram negative (*E. coli*, *P. aeruginosa* and *Acinetobacter sp.*) bacteria, and AgNPs synthesized by *Zizyphus spina christi* L displayed excellent antibacterial activity against all tested bacterial strains at the volume of 100 μL /well (9 μg). AgNPs synthesized by *Zizyphus spina christi* L showed zone of inhibition range from 15 - 24 mm (17 mm for *E. coli*, *S. aureus* 24 mm, *P. aeruginosa*

15 mm, and 23 mm for *Acinetobacter sp.*) (Figure 6 and Table 1). There is no inhibition zone around the well filled with 1 mM AgNO_3 and this indicates the antimicrobial activity is due to biosynthesized silver nanoparticles which were known to have strong antimicrobial activity. The antibacterial activity of AgNPs using this medicinal plant demonstrated that both Gram positive and Gram negative bacteria were inhibited. These results agreed with previous work carried out by Hungund *et al.* [38], Thomas *et al.* [39], and Chitra and Annadurai [40].

The AgNPs impregnated on band aids were tested against previous bacteria, and inhibition zone was recorded. Band aids with AgNPs showed inhibition zone of 19 mm, 18 mm, 15 mm and 16 mm for *S. aureus*, *Acinetobacter sp.*, *P. aeruginosa* and *E. coli*,

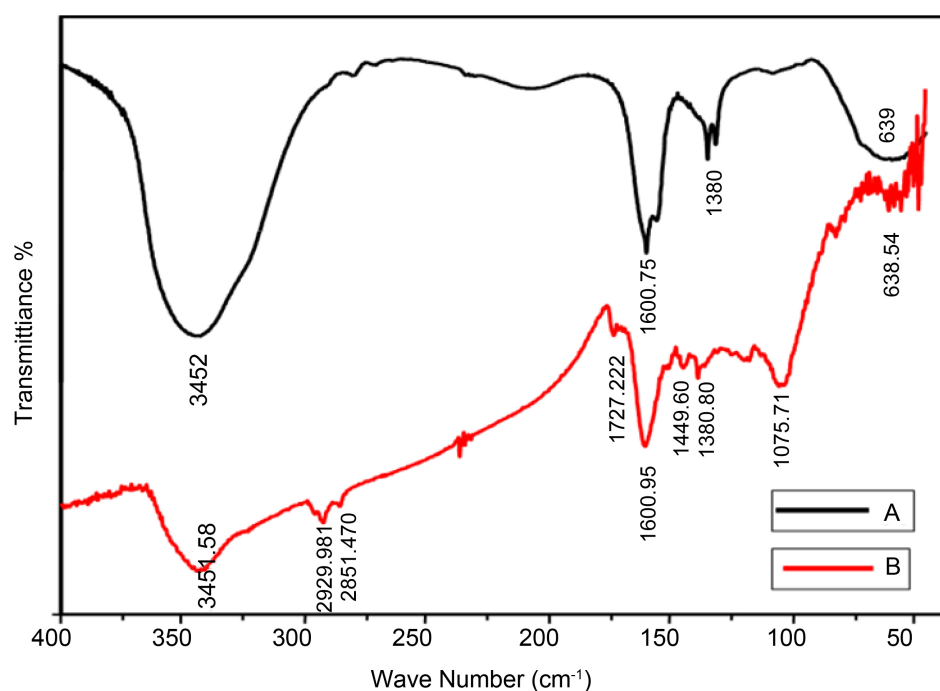


Figure 5. FTIR spectra of *Zizyphus spina christi* L leaves extract (A) and capped AgNPs (B).

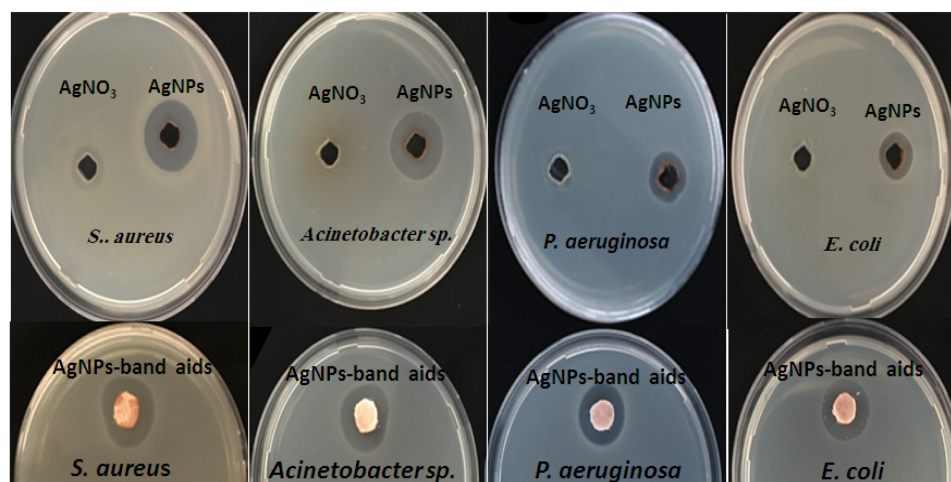


Figure 6. Antibacterial activity of AgNO_3 , AgNPs and AgNPs-band aids against MDR strains of *S. aureus*, *Acinetobacter sp.*, *P. aeruginosa* and *E. coli* patients.

respectively. The efficacy of band aids with AgNPs against pathogens is given in **Figure 6** & **Table 1**, these band aids with antibacterial efficiency of AgNPs can be used as an antiseptic bandage material for wound dressing against antibiotic-resistant bacteria Gram-positive and Gram-negative was confirmed in our results. Our study was in agreement with Balashanmugam and Kalaichelvan who reported a high antibacterial activity of green synthesis silver nanoparticles on cotton cloth [41].

The MIC of AgNPs from *Zizyphus spina christi* L leaves extract was found to be 46, 48, 63 and 59 µg/ml against *S. aureus*, *Acinetobacter* sp., *P. aeruginosa* and *E. coli* strains. Also the MBC of AgNPs from *Zizyphus spina christi* L leaves extract was found to be 57, 61, 91 and 82 µg/ml against *S. aureus*, *Acinetobacter* sp., *P. aeruginosa* and *E. coli* strains (**Table 2**). These findings support the previous reports of antibacterial activity of silver nanoparticles against *S. aureus* MRSA, *Acinetobacter*, *P. aeruginosa* and *E. coli* [42] [43] [44].

In our study, Biosynthesized AgNPs with small size exhibited strong antibacterial activity against tested bacteria. Previous researches showed that antibacterial activity of AgNPs depended on size of nanoparticles [45] [46]. Smaller nanoparticles were found to be optimal against pathogenic bacteria [47]. These nanoparticles exhibited superior ability to penetrate bacteria, interact with membrane and cause damage which may lead to cell death, also silver nanoparticles can be uncouple respiratory electron transport from oxidative phosphorylation, which inhibits respiratory chain enzymes and DNA damage [44] [45] [46] [47] [48]. The results displayed triangle and cubic AgNPs which exhibited highest antibacterial activity, this finding was agreeing with other studies [49]. *S. aureus* was more sensitive to AgNPs than others bacteria used in this study while *P. aeruginosa* was more resistant to AgNPs than others bacteria. For example, MBC of AgNPs against *P. aeruginosa* was 90 µg/ml while it was 57 µg/ml against *S. aureus* (**Table 2**), so there were difference in minimum bactericidal concentration of

Table 1. Diameter of zone of inhibition by biosynthesized AgNPs & AgNPs-cloth against Multi drug resistant pathogenic bacteria.

Strains	ZOI (mm) by AgNPs	ZOI (mm) by AgNPs-band aids
<i>S. aureus</i>	24	1
<i>Acinetobacter</i> sp.	23	18
<i>P. aeruginosa</i>	15	15
<i>E. coli</i>	17	16

ZOI: Zone of Inhibition.

Table 2. The minimum inhibitory concentration of AgNPs (MIC) and Minimal Bactericidal Concentration (MBC) toward Multi drug resistant bacteria.

Strains	MIC (µg/ml)	MBC
<i>S. aureus</i>	45	57
<i>Acinetobacter</i> sp	49	61
<i>P. aeruginosa</i>	63	90
<i>E. coli</i>	59	82

AgNPs against different bacteria isolate, that means the antibacterial activity of AgNPs depend on the concentration of AgNPs against each bacteria and this was agreement with previous studies [50] [51]. The antibacterial activity of silver nanoparticles could be related to several Factors (size, shape and concentration of MIC and MBC) and we take that in our consideration during synthesis of nanoparticle for clinical use.

Finally, this simple and rapid process for the biosynthesis of silver nanoparticles using aqueous extract of *Z. spina christi*, hemonstrated several advantages with low cost and compatibility for the bioorganic compound capped AgNPs to be used against multi drug resistant bacteria in medical filed.

4. Conclusion

The present work was focused on the development of a green, rapid and simple method for the synthesis of AgNPs using Saudi medicinal plant. Silver nanoparticles prepared in this process were fast, low cost, eco-friendly, and could be potentially applied in variety of solutions for different safe medical application. Biosynthesis of silver nanoparticles using *Zizyphus spina christi L* was prepared by rapid method within 30 mints; the XRD analysis confirmed that the synthesized AgNPs had a face centered cubic phase (fcc). TEM analysis showed that the nanoparticles were hexagonal in shape with different size ranging from 21.5 to 59.67 nm under optimum conditions. The biomolecules analysis showed that alcohols, phenols, polysaccharides and proteins were important for biosynthesis of AgNPs as active molecules dissolved in water extract of *Zizyphus spina christi*. Moreover, the synthesized AgNPs and their incorporation in band-aids enhanced the therapeutic efficacy of band and promoted a very good wide spectrum antibacterial material as bioactive band for wound dressing, especially against multi drug resistant bacteria.

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